Journal of Chromatography 80 (1973) 89–100

CHROM. 6659

ANALYSIS OF CORTICOSTEROIDS IN MIXTURES BY GRADIENT ELUTION LIQUID CHROMATOGRAPHY

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(First received November 27th, 1972; revised manuscript received February 21st, 1973)

SUMMARY

A method is described for the fractionation of corticosteroids in mixtures by means of column chromatography with small size columns, reduced elution times and continuous monitoring of the effluent with data recording.

As a test mixture, eight natural corticosteroids were chosen in order to reproduce extracts from glands or biological fluids: corticosterone, 11-dehydrocorticosterone, 11-deoxycortisol, cortisone, cortisol, aldosterone, tetrahydrocortisone and tetrahydrocortisol.

Firstly, the influence of the activation of the adsorbent on the separation was studied and the shape and the extent of the gradient were considered. The adsorbents were silicic acids of different structural types, and the gradient components were methanol and chloroform.

Monitoring of the effluent was performed by means of two detectors, acting in parallel: the first was a UV detector and the second was a hydrogen flame ionization detector, part of the effluent being continuously drawn off to the detector.

Conditions for the optimization of the separations are reported and possible analytical applications are discussed.

INTRODUCTION

The separation of adrenocortical steroids by using chromatographic procedures for the analysis of mixtures originating from biological extracts or present in pharmaceutical preparations is still of considerable analytical interest, as is demonstrated by the number of papers and reviews published on the subject.

Limiting ourselves to papers concerning analytical column chromatography with monitoring of the effluent, of importance is the work of Johnson and coworkers[1–8], who used silicic acid columns, of Siggia and Dishmann[9], who used synthetic resin columns, of Murphy[10], who used columns of Sephadex LH-20, and of Graham *et al.*[11], who used partition columns. This type of column was also used by Mollica and Strusz[12], together with modern apparatus for high-pressure liquid chro-

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matography (LC) for the direct determination of corticosteroids in ointments, and Henry *et al.*[13] described the general application of this type of instrumental technique to the analysis of steroids.

In previous papers[14,15], we described the separation of various androgenic, estrogenic and progestational steroids from each other and from the major and minor components of their oil solutions[16], using column chromatography on silicic acid and elution with a gradient of diethyl ether in hexane. As interesting results were obtained by using silicic acid columns and gradient elution, we have now extended our experiments to the fractionation of corticosteroid mixtures.

RESULTS AND DISCUSSION*

Experiments with Bio-Rad silicic acid, 325 mesh

Influence of the water content. On the basis of our previous results[14,15] and from some preliminary assays, it was considered necessary to establish the water content to which silicic acid should be brought after the activation treatment, in order to obtain a material that is capable of giving reproducible results and also to obtain a separation of the various steroids examined that is as satisfactory as possible.

Materials adjusted to the following water contents after activation were compared: (1) no addition (maximum activation); (2) up to 8.5% of the total weight; (3) up to 15% of the total weight; and (4) up to 20% of the total weight. The effect of the different water contents, in terms of retention volumes, is shown in Table I. It can be seen that the retention volumes first increased and then decreased, when the added water content increased from 0 to 20%.

TABLE I

EFFECT OF WATER CONTENT OF SILICIC ACID ON RETENTION VOLUMES

Values are retention volumes (V_R, ml) obtained directly with the LC detector, using the same column size (560 mm height, 6 mm diameter), gradient (concave gradient C_1), flow-rate (1 ml/min) and sample (500 μg).

Substance	*Water content (%)*			
	Minimum	*8.5*	*15*	*20*
11-Dehydrocorticosterone	37.5	63.0	37.0	22.5
Corticosterone	57.0	87.0	59.0	33.0
Cortisone	67.0	105.0	78.8	61.0
Cortisol	92.0	120.0	96.3	91.0
Tetrahydrocortisone	104.0	134.0	110.6	97.0
Tetrahydrocortisol	129.0	163.0	134.8	114.0

From these results, the most useful material seems to be silicic acid containing 15% of added water and that at maximum activation in terms of the absolute values of the retention times and their differences for the compounds to be separated.

Influence of the column dimensions. The maximal permissible pressure drop with our apparatus was 500–600 p.s.i. and we therefore examined columns of different dimensions, using them under conditions of about the maximal flow-rate of the

* For explanation of compound codes, see Materials and methods.

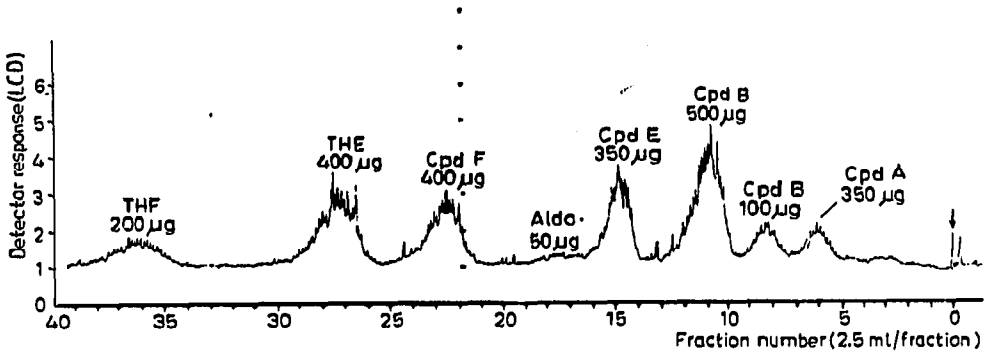

Fig. 1. Chromatogram of a reference steroid mixture obtained with a column of 1 m length and 2 mm diameter. Operating conditions: silicic acid plus 15% of added water; concave gradient C_1; flow-rate 0.5 ml/min; amount of sample 2.350 µg in total.

effluent compatible with the apparatus. Fig. 1 shows a chromatogram obtained under the best resolution conditions (a column of 1000 mm length and 2 mm diameter) with the effluent being monitored by means of an LC, detector, which also detects tetrahy-compounds.

In Table II are compared the resolutions between five pairs of compounds using four different columns, and the times required for each separation are also reported.

TABLE II

COMPARISON OF RESOLUTIONS* FOR FIVE PAIRS OF SUBSTANCES FROM LC DETECTOR RECORDING, WITH DIFFERENT CONDITIONS OF COLUMN SIZE AND FLOW-RATE AND THE SAME CONDITIONS FOR THE ADSORBENT (SILICIC ACID WITH 15% WATER CONTENT), GRADIENT (CONCAVE GRADIENT C_1) AND AMOUNT OF SAMPLE (350 µg)

Column size		*Flow-rate*	$R_{Cpd\ A,Cpd\ B}$	$R_{Cpd\ B,Cpd\ E}$	$R_{Cpd\ E,Cpd\ F}$	$R_{Cpd\ F,Cpd\ THE}$	$R_{Cpd\ THE,Cpd\ THF}$	*Duration*
l (mm)	*I.D. (mm)*	*(ml/min)*						*(min)*
600	6	1.00	1.16	0.96	1.00	0.85	1.18	160
600	4	0.75	1.11	1.10	1.52	0.91	1.40	155
500	2	0.50	1.30	1.10	1.52	1.04	1.39	170
1000	2	0.50	1.50	1.18	2.30	1.04	1.70	195

* $R_{1,2} = 2\,(V_{R2} - V_{R1})/(W_1 + W_2)$

Influence of the type of gradient. Two basic types of gradient of methanol in chloroform were examined: concave and linear. For both types, the variation of the concentration of the more polar component with the eluent volume can be described by the following general equation according to Wren[17]:

$$C_V = C_R - (C_R - C_M)\,(1 - v/V_R + V_M)^p$$

were $p = 1$ for the linear gradient and $p < 1$ for the concave gradient. Two sets of curves for the two types of gradient are shown in Fig. 2.

In order to help in choosing a convenient type of gradient, we obtained experimentally a graph of the volume of eluent (abscissa) against the concentration of methanol in the eluent corresponding to the elution of a particular steroid obtained with different gradients (ordinate). The resulting curves represent the variation of the

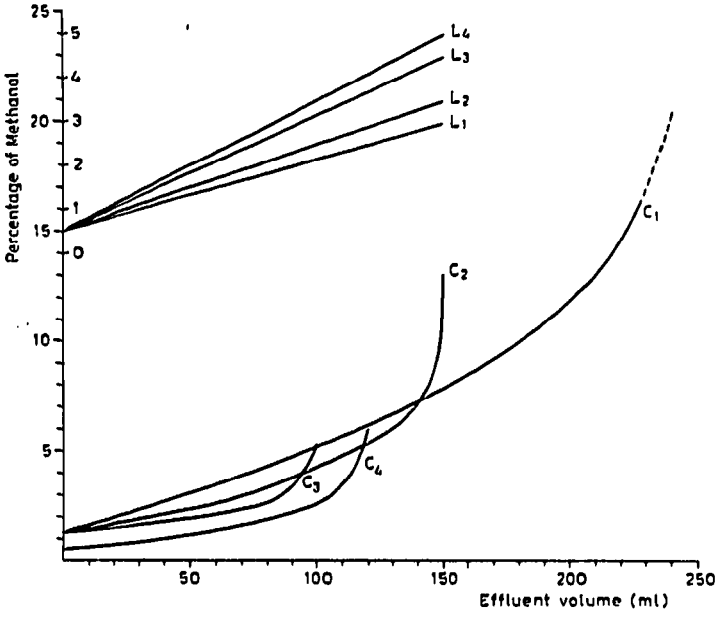

Fig. 2. Gradient curves corresponding to the two types of gradient used.

Concave (C) gradients

	V_M	C_M	V_R	C_R
C_1	200	1.3	50	33.0
C_2	120	1.3	30	13.1
C_3	80	1.3	20	5.6
C_4	96	1.3	24	6.0

Linear (L) gradients

	V_M	C_M	V_R	C_R
L_1	75	0.5	75	3.0
L_2	75	0.5	75	3.5
L_3	75	0.5	75	4.5
L_4	75	0.5	75	5.0

elution volume with the concentration of methanol in the eluent for each substance. The curves are nearly linear (Fig. 3) and have a negative and relatively small angular coefficient, and confirm that there is a narrow range of methanol concentrations necessary for elution to be carried out with reasonable volumes of eluent. With a graph of the type shown in Fig. 3, it is possible to calculate theoretically the conditions for the optimal separation with a given type of column simply by superimposing a gradient curve, $C_{MeOH} = f(V_e)$, that intercepts the elution curves of the different steroids at the most convenient points.

For practical reasons, we limited the work to the use of gradients of concave or linear types which can be obtained with the simple experimental facilities described in earlier papers[14–16].

Experiments with Corasil II and Porasil A

On the basis of the satisfactory selectivity obtained with silicic acid, we decided to examine two different types of silicic acid with different structural and granulometric characteristics: Corasil II*, which consists of glass cores with a surface of porous silicic acid of controlled thickness and a diameter of 37–50 μm; and Porasil A*, a silicic acid with porous, rigid, spherical particles, 37–75 μm in diameter, with a

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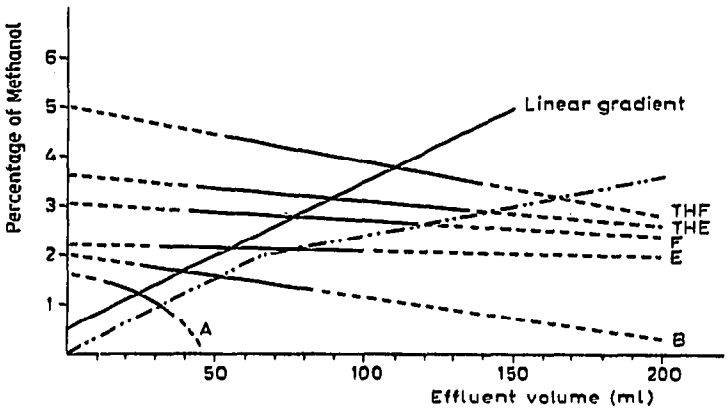

Fig. 3. Elution curves for different steroids showing the range of methanol concentrations corresponding to reasonable volumes of effluent. The continuous lines indicate the range of effluent volumes used for the measurements; the "linear gradient" line shows how a linear gradient curve (C_{MeOH} in the effluent *vs.* the effluent volume) intercepts the elution curves for the different steroids, indicating their retention volumes on the abscissa. The intercepts of the broken lines with the steroid elution curves indicate, on the abscissa, the upper limits of the practically permitted elution volumes.

controlled pore size and surface area.

The gradients and column dimensions used were the most satisfactory obtained with silicic acid. In Table III, the results obtained in comparable experiments with the above two materials and with silicic acid are reported. The following can be seen from Table III.

(*a*) The flow-rates obtainable with a certain pressure drop for both Corasil II and Porasil A were higher than those for silicic acid, thus enabling longer columns to be used or the analysis time to be shortened.

(*b*) The resolution on Corasil II columns of 1 m length was poor between compounds A and S, and this resolution, as well as those for the other pairs of steroids, remained practically unchanged when Corasil II with 0.5% of added water was used.

Porasil A gave satisfactory resolution for the pairs of steroids studied, except for those involving aldosterone, which gave very poor peaks, badly resolved from those of compound E.

Good results were obtained in this comparison experiment with a column of Bio-Rad silicic acid, 325 mesh, 300 mm height and 2 mm diameter, using a simple linear gradient (Type L_1), with a flow-rate of 1 ml/min and a separation time of *ca.* 120 min (see Fig. 4). The results showed that, from the point of view of separation, it is equally convenient to work with silicic acid at maximum activity or with silicic acid containing 15% of water, and therefore, in order to simplify the preparation and to facilitate re-use of the column, it was decided to use the first type of material. Applications of this type of column to quantitative analysis are reported later in Table V.

(*c*) Corasil and Porasil columns as well as those of Bio-Rad silicic acid used under the above conditions can be re-used after a short equilibration with chloroform.

(*d*) In analytical applications, it is stressed that with all of the materials examined

TABLE III

COMPARISON OF RESOLUTION VALUES* IN EXPERIMENTS WITH SILICIC ACID, CORASIL II AND PORASIL A ON SIX PAIRS OF SUBSTANCES FROM UV DETECTOR RECORDING

The same conditions were used for the amount of sample (reference mixture, 356.6 μg) and the gradient (linear L_1).

Adsorbent	*Column size*		*Flow-rate*	*Resolution*						*Duration*
	h (mm)	*I.D. (mm)*	*(ml/min)*	*Cpd Q/Cpd A*	*Cpd A/Cpd S*	*Cpd S/Cpd B*	*Cpd B/Cpd E*	*Cpd E/Cpd Aldo*	*Cpd Aldo/Cpd F*	*(min)*
Silicic acid, max. activation	300	2	1.0	3.8	1.1	1.0	1.5	1.2	1.1	110
Silicic acid, max. activation	500	2	1.0	4.0	1.1	1.1	1.6	1.2	1.2	130
Corasil II, max. activation	500	2	1.0	–	–	–	1.6	1.7	0.8	60
Corasil II, max. activation	1000	2	1.0	5.0	0.7	1.5	1.7	1.8	1.0	80
Corasil II, 0.5% added water	1000	2	1.0	5.5	0.8	1.9	1.9	2.2	1.4	75
Porasil A, max. activation	500	2	1.0	3.0	1.0	1.3	1.3	–	–	95
Porasil A, max. activation	1000	2	1.5	3.6	1.0	1.3	1.5	–	–	90

* $R_{1,2} = 2(V_{R2} - V_{R1})/W_1 + W_2$.

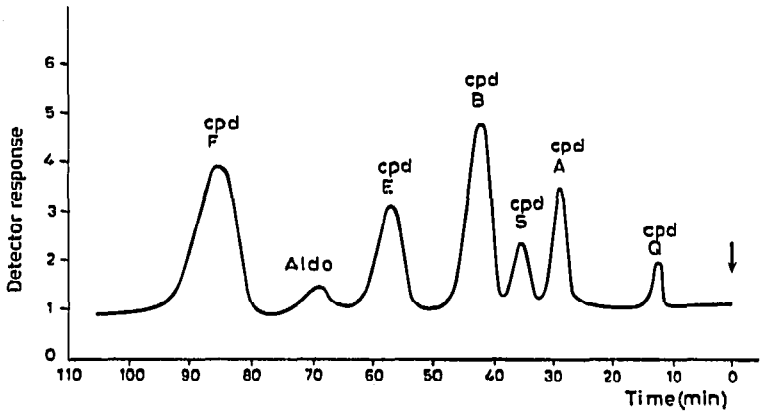

Fig. 4. Chromatogram of a reference steroid mixture obtained with a column of 300 mm length and 2 mm diameter. Operating conditions: silicic acid at maximum activity; linear gradient L_1; flow-rate 1 ml/min; amount of sample 278 μg.

the form of the peaks is regular and generally symmetrical, which is of advantage when direct determinations by peak area must be carried out.

Quantitative analysis

For the quantitative analytical investigations we studied (1) a procedure of analysis carried out after obtaining the chromatogram, and (2) a direct analytical procedure carried out on the chromatogram by peak area measurements.

In Table IV the results obtained with the first type of procedure in replicate analyses of different types of samples are given. In the case of sample (a), the separation between the various compounds was complete and the recoveries were within the range 90–100%, with good correspondence between parallel samples; the latter evidence indicated the good reproducibility of the chromatographic separation process.

Another point of interest in our findings is reflected by the recent observation of Lamontagne and Johnson[6] that in the chromatography of adrenocorticosteroids on silicic acid, comparing adsorbents of different origins, only a few of them and only under suitable conditions gave satisfactory recoveries (losses amounted to as much as 50%) and separations of good quality.

The analysis of sample (b) is reported as an example of the application of the procedure to a natural mixture of corticosteroids. The results are considered to be satisfactory, since it is possible to analyze a certain number of basic compounds in the extract, detected by examination of the effluent under UV light and further pairs identified by determining retention volumes with the LC detector and successive thin-layer chromatographic (TLC) examination.

The application of the present chromatographic procedure to the specific problem of the analysis and detection of steroids in cortical extracts will be described in detail in another paper[18].

For the second type of analytical procedure, we used a mixture of corticosteroids of known composition, reported in Table V. The mixture had a total steroids

TABLE IV

RESULTS OF QUANTITATIVE ANALYSIS ON THE ELUATE

Recovery is reported as the percentage of the single (sample a) or of the total (sample b) amounts chromatographed.

Sample	*Amounts chromatographed (μg)*	*Recovered amounts of compounds (%)* *										
		X_0 (1–3)	*A (4–6)*	*S (7–9)*	*B (10–13)*	*E (14–16)*	*Aldo (17–22)*	*F + X_1 (23–28)*	*THE + X_2 (29–33)*	*α-THF (34–39)*	*THF*	*Total recovery*
(a) Mixture of Cpds B, E, F, THE, THF 250 μg for each Cpd)	1250	–		–	94.4**	100.2**	–	93.5**	94.9***	–	90.9***	–
(a') As (a)	1250	–	–	–	94.1**	96.6**	–	96.4**	94.5***	–	90.5***	–
(b) Adrenal extract	1010**	9.6	18.0	7.6	13.3	13.2	5.1	21.3	5.4	1.9	–	95.4**
	1027***	3.7	15.3	5.6	15.6	12.4	4.4	28.4	2.1	4.1	–	91.6***
(b') As (b)	1010**	6.0	17.9	7.2	14.7	14.0	5.1	22.6	3.9	2.7	–	94.1**
	1027***	1.7	16.1	5.1	16.8	13.7	4.2	29.9	1.8	4.4	–	93.7***

* Fraction numbers are given in parentheses.

** UV analysis.

*** Blue tetrazolium colorimetric analysis.

TABLE V

RESULTS OF QUANTITATIVE ANALYSIS BY PEAK AREA MEASUREMENTS

Compound	*Amount in sample (%)*	$R^* \pm S.D.$	*Relative standard deviation (%)*
Q	2.15	1.159 ± 0.118	± 10.2
A	11.40	0.916 ± 0.051	± 5.6
S	4.84	1.442 ± 0.072	± 5.0
B	27.12	0.877 ± 0.040	± 4.6
E	15.39	0.982 ± 0.037	± 3.8
F	31.75	–	–
Aldo	1.61	1.356 ± 0.122	± 9.0
Tetrahydrocompounds	2.47	–	–

* R = Area Cpd i/Area Cpd F × Q Cpd F/Q Cpd i, as mean value from nine chromatographic analyses with scalar amounts of the sample ranging from 279 to 650 μg.

content of 3.72 mg/ml and five replicated scalar aliquots of 75–175 μl, ranging from 279 to 651 μg, were chromatographed in parallel. For each chromatogram the relative areas of the peaks, corresponding to the various compounds, were determined. In order to evaluate the reproducibility of the procedure with different amounts of sample of compound i placed on the column, values of the relationship Area Cpd i/Area Cpd F were calculated, taking compound F as internal standard, and relative response factors, R, currently used in gas chromatographic (GC) analysis[19] as defined by the expression

$$R = \text{Area Cpd i/Area Cpd F} \times Q \text{ Cpd i}/Q \text{ Cpd F}$$

were calculated together with the corresponding standard deviations. The results were generally good, as shown by the standard deviations.

From the results obtained, it appears that the procedures described give, in a relatively short time, good and reproducible separations, which are advantageous since (*a*) they allow the use of active materials that are easily obtained and standardized; (*b*) they require the use of linear or concave gradients that can be produced with simple and inexpensive apparatus; and (*c*) they permit the application of more than one analytical procedure to the eluate for the analysis and identification of the separated compounds or else they permit the analysis of the chromatogram in accordance with the criteria of high-speed, high-pressure LC.

MATERIALS AND METHODS

Solvents and materials for chromatography

Methanol and chloroform of analytical grade were distilled twice before use. In order to obtain good, comparable and reproducible separations, it is necessary to use chloroform with a certain initial methanol content, which, at this particular level, is advantageous for the separation. Instead of the low starting levels of methanol in the mixtures, equivalent ethanol–chloroform mixtures can be used with no differences in the chromatographic behaviour; this greatly simplifies the preparation of the solvents. The ethanol content of chloroform was determined by means of a GC procedure[20] on a column of dimethylstearamide (Hallcomid M-18, Hewlett-Packard) on Chromosorb W, 80–100 mesh, using *n*-butanol as internal standard; only two concentra-

tions were chosen, 0.5 and 1.3%, these being convenient for separation purposes.

Bio-Rad silicic acid, 325 mesh, for chromatographic purposes following Hirsh and Ahrens, was conditioned for water content as follows. The material was heated to 125° for 8 h in an oven, cooled in a desiccator and w ml of water were added to $100 - w$ g of product in order to obtain a conventional w % of water; w ranged from 0 to 20%, as indicated in Table I. Porasil and Corasil II were used at maximum activity, as described by the manufacturer.

Chromatographic columns

The following types of columns were used:

(*a*) LKB glass column, Type 4200, 6 mm in diameter and 600 mm in length;

(*b*) Pye glass column, 4 mm in diameter and 600 mm in length;

(*c*) Chromatronix glass columns, 2 mm in diameter and 300, 500 or 1000 mm in length.

All the columns were provided with head injectors, using for type (*a*) a device made in our laboratory and for types (*b*) and (*c*) the appropriate injectors supplied by the column manufacturer. Connections between the column inlet and the preceding parts of the instrument (gradient vessels and pumps) were made of PTFE tubes, *ca.* 1/16 in. O.D. and 0.031 in. (0.8 mm) I.D., while between the column outlets and the monitoring instruments the tubes were of 0.012 in. (0.3 mm) I.D. and 1/16 in. O.D.

Samples

The following steroids of chromatographic purity were used: 11-deoxycorticosterone (compound Q); 11-dehydrocorticosterone (compound A); 11-deoxycortisol (compound S); corticosterone (compound B); cortisone (compound E); aldosterone (compound Aldo); cortisol (compound F); tetrahydrocortisone (compound THE); tetrahydrocortisol (compound THF) and 5α-tetrahydrocortisol (compound 5α-THF).

Solutions of the above compounds in ethanol at a concentration of 1 mg/ml were prepared and stored in a cold room. For the experiments, calculated amounts of the ethanolic solutions were taken, combined and evaporated under nitrogen in order to obtain mixtures of corticosteroids of known composition; an appropriate volume of chloroform was added to the residue and the final solution obtained was used for the analysis.

For the cortical extract to be used in the recovery experiments shown in Table IV for sample (b), the starting material was an aqueous–alcoholic solution (90:10, v/v) of the corticosteroid extract from bovine or porcine glands, obtained by the Cartland and Kuizenga procedure[21]. The steroids were extracted with chloroform, purified and named as described by Cavina *et al.*[22].

The introduction of the samples on to the column was performed by means of gas-tight microsyringes, in volumes of 150 μl or less.

Feeding system for the column

This system was described in detail earlier[16]. Concerning the optimization of the gradient in order to obtain the best separation for a given mixture of substances, it is theoretically possible to have a curve, according to the equation described by Wren[17]:

$$C_V = C_R - (C_R - C_M)(1 - v/V_R + V_M)^p \quad (1)$$

where $p = V_R/V_M = R_1/R_2 - R_1$ and R_1 and R_2 are flow-rates of V_R to V_M and of V_M to the column, respectively, which passes through a number of points of coordinate v,C_v, equal to the number of independent variables, which in our case are C_R, C_M, p, V_T, since the following relationships are valid for V_R, V_M, V_T and p:

$$p = V_R/V_M \quad (2)$$

$$V_T = V_R + V_M \quad (3)$$

The optimization could therefore be carried out for a maximum of four values, writing the four variations of eqn. 1 and obtaining the four unknowns, C_R, C_M, p and V_T. This is laborious and of little use for the analyzed mixtures, for which we successfully used these considerations only in order to obtain the concentration data for the best separation compatible with a particular type of gradient ($p = 1/4$, concave; or $p = 1$, linear) in a pre-determined elution volume, V_T.

For example, it is desirable to elute a mixture of corticosteroids, as described in the text, by a concave gradient $p = 1/4$ in 100 ml, the effluent flow-rate, R_2, being 0.5 ml/min and the initial content of methanol in chloroform in the mixing vessel being 1.3% ($C_M = 1.3\%$).

The total volume V_T of the gradient can safely be fixed at 150 ml, taking into account a certain degree of tailing of the peak. The values of C_M, p and V_T being already known, only C_R remains to be established. It was found from Fig. 3 that the elution volume v of THF (which will be the last compound to be eluted) is 100 ml, and at this volume a concentration C_V of 3.9% of methanol in the effluent is reached; by introducing this condition in eqn. 1, we obtain $C_R = 12.1\%$.

Analysis of the eluates

The UV detector was a double-beam instrument (Photochrom I, Rastelli, Rome) equipped with quartz cells of 10 mm optical path, less than 0.1 ml in volume, equipped with a Hilger and Watts diffraction-grating monochromator fixed at 240 nm and a differential amplifier with two photomultipliers. The signal was passed to a 5 mV full-scale recorder, and the recorder signal was directly proportional to the concentration of the solution for a limited range, although it enabled reasonably linear calibration curves of the amount injected *versus* response to be constructed.

The output from the sample cell was connected by means of a splitting device, already described[16], to a Barber and Colman Model 5400 LC detector. The splitting ratio was adjusted to 6% of the column outlet, operating at a flow-rate of 1 ml/min, and was proportionally increased for lower flow-rates. The operating conditions of the LC detector were as described earlier[16].

The overflow from the splitter was sent to a fraction collector and fractions of 5 or 2.5 ml of the effluent were taken. Qualitative control for identification purposes in the eluates was carried out by TLC with convenient aliquots of the fractions[23,24].

The first type of quantitative analysis was performed on columns 1000 mm in length, filled with silicic acid containing 15% of water, and the elution was carried out with a concave gradient (C_2) at a flow-rate of 0.5 ml/min. The eluate was collected in fractions of 2.5 ml (40–50 fractions) and the fractions corresponding to the various peaks were grouped, in order to perform the quantitative determinations, as follows: (*a*) a UV absorption spectrometric method at 240 nm with a Beckman DU-2 instrument; (*b*) a blue tetrazolium colorimetric method following the procedure of Nowaczynsky *et al.*[25].

The eluates corresponding to the different peaks were evaporated, the residues re-dissolved in spectroscopic grade ethanol and adjusted to a volume of 10 or 25 ml with ethanol as appropiate for direct UV analysis. For the calculations, the absorptivity values were taken as follows: compound F, 44.5, 242 nm; compound E, 44.0, 238 nm; compound B, 47.8, 240 nm. For the blue tetrazolium analysis, convenient aliquots were taken from the ethanolic solutions, concentrating them with a nitrogen flow in a water-bath if necessary; calibration curves were obtained for compounds F, THE and THF.

In the analysis of cortical extracts, reference was made to compound F for both UV and tetrazolium determinations.

The second type of quantitative analysis (based on peak areas) was performed by injecting replicate amounts of five scalar aliquots of a steroid reference mixture, the composition of which is reported in Table V, on to columns 300 mm in height and 2 mm in diameter filled with silicic acid at maximum activity and eluted with a linear gradient (L_1) at the flow-rate of 1 ml/min. The areas of the peaks were calculated as the product of the peak height and the width at half-height.

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